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Immunocytochemical and Cytochemical Localization of Photosystems I and II[1]

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ABSTRACT

Cytochemical and immunocytochemical methods were used to localize photosystems I and II in barley (*Hordeum vulgare* L. cv Himalaya) chloroplasts. PSI activity, monitored by diaminobenzidine oxidation, was associated with the lumen side of the thylakoids of both grana and stroma lamellae. The P_{700} chlorophyll *a* protein, the reaction center of PSI, was localized on thin sections of barley chloroplasts using monospecific antibodies to this protein and the peroxidase-antiperoxidase procedure. Results obtained by immunocytochemistry were similar to those of the diaminobenzidine oxidation: both grana and stroma lamellae contained immunocytochemically reactive material. Both the grana and stroma lamellae were also labeled when isolated thylakoids were reacted with the P_{700} chlorophyll *a* protein antiserum and then processed by the peroxidase-antiperoxidase procedure. PSII activity was localized cytochemically by monitoring the photoreduction of thiocarbamyl nitroblue tetrazolium, a reaction sensitive to the PSII inhibitor, DCMU. PSII reactions occurred primarily on the grana lamellae, with weaker reactions on the stroma lamellae.

Despite the many ultrastructural and biochemical studies of thylakoid membranes of the higher plant chloroplast (1–5, 16), the precise locations of PSI and II in these membranes have not been firmly established. Current models of thylakoid organization (4, 16) suggests that both PSI and II are found in the stroma and grana lamellae. Recently, however, Andersson and Anderson and co-workers (1, 2) have suggested that PSI is virtually absent from appressed regions of the thylakoid and that only the PSII reaction center and light-harvesting pigments are found in the appressed membranes of the grana stack. In an effort to reconcile the differences between these models based upon membrane fractionation techniques, we have utilized cytochemical and immunocytochemical procedures to localize the two photosystems *in situ* in higher plant chloroplasts. PSI was localized by the use of DAB[3] oxidation and by immunocytochemical localization of the P_{700} Chl *a* protein, the reaction center protein of PSI. PSII was localized cytochemically by monitoring the reduction of TCNBT. Our data indicate that PSI and II are present both in the grana and stroma lamellae with a preponderance of PSII localized in the grana lamellae.

MATERIALS AND METHODS

Plant Material. Seeds of *Hordeum vulgare* L. cv Himalaya were germinated in the dark in 2 mM $CaSO_4$ (pH 5.7). Resultant plants were grown in vermiculite for 6 d in the dark, watered daily with 2 mM $CaSO_4$. Material for use in the studies described below was collected after 24 h of 400 $\mu E \cdot m^{-2}\ s^{-1}$ PAR light exposure. Samples for cytochemistry and immunocytochemistry were obtained from the same plants so that direct comparisons could be made between the various techniques.

Cytochemistry. Small leaf segments (1-mm^2 sections taken approximately 5–10 mm from the tip of the primary leaf) were fixed in 2% (w/v) paraformaldehyde in 0.10 M phosphate buffer (pH 7.2) for 1 h at 0 to 4°C in the dark and were washed 3 times over 2 h in 5% (w/v) RNase-free sucrose in 0.10 M phosphate buffer (pH 7.2). The material was then pre-incubated for 1 h at 0 to 4°C in the dark in the appropriate reaction mixture (1 mg/ml DAB in phosphate buffer or 1 mg/ml TCNBT in 5% dimethyl sulfoxide in phosphate buffer) to allow for uptake of the reagent. The specimens were then transferred to a fresh reaction medium and incubated under 400 $\mu E \cdot m^{-2}\ s^{-1}$ (PAR) for 1 h at 22°C. Control specimens were incubated in the dark, in the absence of the staining reagent, or with the addition of 10^{-6} M DCMU. After incubation, the specimens were washed in two changes of phosphate-buffered sucrose (15 min each, in the dark) to reduce thylakoid swelling and postfixed in 1% (w/v) OsO_4 in 0.10 M cacodylate buffer (pH 7.2) for 1 h at 0 to 4°C. Specimens were washed twice in cold (0–4°C) distilled H_2O, dehydrated in acetone (to 70% acetone at 0–4°C), and embedded with Ladd's Ultralow viscosity resin (Ladd Inc., Burlington, VT). Gold-silver sections were observed without poststaining with a Hitachi HU-11 C electron microscope. Further discussion of these two cytochemical techniques can be found in Reference 21.

P_{700} Chl *a* Protein Antiserum. The P_{700} Chl *a* protein was isolated from barley chloroplast membranes by preparative SDS gel electrophoresis (23, 24). Protein was passively eluted from the preparative gels and absorbed to alum (9). New Zealand White female rabbits were inoculated with 200 μg protein in Freund's complete adjuvant; injections were made every 2 weeks

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[3] Abbreviations: DAB, 3,3′-diaminobenzidine; TCNBT, thiocarbamyl nitroblue tetrazolium; TMBZ, tetramethylbenzidine; PAP, peroxidase-antiperoxidase.

subsequently, with 100 to 200 μg protein in Freund's incomplete adjuvant. Animals were bled 8 to 10 d after booster injections. Monospecificity and other properties of the antibodies for the apoprotein of the P_{700} Chl *a* protein are described in detail elsewhere (23).

Immunocytochemistry. *Postembedding Staining on Thin Sections.* Small leaf pieces were fixed in 2% (w/v) paraformaldehyde, 2% (v/v) glutaraldehyde in 0.050 M cacodylate buffer (pH 7.2) for 1 h at 0 to 4°C. After two 0 to 4°C H_2O washes, the material was dehydrated in acetone and embedded in Ladd's Ultralow viscosity resin. Gold-silver sections were mounted on nickel grids and subjected to the PAP immunocytochemical procedure of Sternberger (19), as follows: 3% H_2O_2, 5 min; 0.1% goat serum, 5 min; 1:800 dilution of rabbit antisera to the P_{700} Chl *a* protein, 48 h at 0 to 4°C and 2 h at 22°C; 0.1% goat serum, 5 min; 0.066 mg/ml PAP (Polysciences Inc., Warrington, PA), 5 min; 0.0125% DAB and 0.0025% H_2O_2, 3 min; 2% (w/v) OsO_4, 30 min. All dilutions were carried out in 0.1 M Tris, 0.5 M saline (pH 7.6) and the DAB reaction was carried out in 0.05 M Tris-HCl(pH 7.6). Immunocytochemical reactions were carried out at 22°C unless otherwise noted. Controls substituted pre-immune rabbit sera for the P_{700} Chl *a* protein antisera.

Pre-embedding Staining on Isolated Thylakoid Membrane. Leaves were harvested and ground in a Waring blender (three bursts, 10 each) in ice-cold 0.40 M sorbitol in 0.10 M Tricine-NaOH (pH 7.6). The brei was strained through Miracloth and centrifuged at 500*g* for 5 min to remove debris. The supernatant was centrifuged at 3000*g* for 15 min and the pellet was used as a crude source of chloroplast fragments. The pellets were fixed for 30 min in 3% (v/v) glutaraldehyde in 0.10 M cacodylate buffer (pH 7.2 at 0.4°C and washed 4 times (15 min each) with 0.10 M phosphate buffer (pH 7.2) supplemented with 20 mM lysine to remove excess glutaraldehyde (14).

The washed thylakoids were then subjected to the same series of reagents as used for the PAP thin section staining (above). At each stage, the thylakoid preparations were mixed on a Vortex mixer and repelleted at 13,000*g* for 15 min to facilitate exchange of solutions. Because of the fragility of the thylakoid preparations all steps of the procedure were carried out at 0 to 4°C and incubation in the P_{700} Chl *a* protein antisera was limited to 2 h at 0 to 4°C. Longer incubations lead to disruption of the thylakoids, preventing the distinction between thylakoids and contaminating parts of other organelles. After incubation in the immunocytochemical reagents, the thylakoids were processed for microscopy as described above. Sections were observed without poststaining. Controls substituted pre-immune sera for the P_{700} Chl *a* protein antisera.

RESULTS AND DISCUSSION

Cytochemical Studies. Numerous authors (7, 15, 17, 18, 21, 22, 25) have used the photooxidation of DAB as a sensitive probe to localize PSI. This reaction is insensitive to inhibitors of PSII activity (15, 17) and is completely dependent upon light (17). Mutants deficient in PSI activity (22) or etioplasts which have not developed photosynthetic competence (25) do not photooxidize DAB. In mesophyll cells of barley, DAB photooxidation was observed in both the stroma and grana lamellae of the chloroplast (Fig. 1A). No reaction is noted on the chloroplast envelope (Fig. 1A) and this lack of envelope reaction allows one to compare easily the density of an unreacted area to the reaction product. The discrete distribution of the reaction product suggests that this distribution occurs as the result of true reactions and is not due to precipitate migration. At higher magnification, reaction product appears to be localized on the lumen side of the thylakoid (Fig. 1A, inset) rather than the stroma surface, suggesting that the donation of electrons from DAB to PSI occurs on this thylakoid surface rather than on the stroma side. A similar observation was made by Porat *et al.* (18). The localization of PSI on the outer surface of the thylakoids by Imaizumi and Hiraoka (7) may have been due to the addition of methyl viologen to their reaction medium. Superoxide, produced by the reduction of molecular O_2 by reduced methyl viologen, can cause the oxidation of DAB in the presence of divalent metal cations (10). Controls incubated in the absence in the dark did not show the precipitation of reaction products (Fig. 1B).

Reduction of TCNBT to its insoluble osmiophilic formazan was readily detectable as electron-dense deposits along the surface of both the grana and stroma lamellae (Fig. 1C). TCNBT photoreduction is completely eliminated by the PSII inhibitor DCMU, indicating the specificity of this reaction (Fig. 1D). Some thylakoid dilation was obtained due to the addition of the inhibitor to the reaction mixture. The reaction is most intense in the partition region of the grana stacks (Fig. 1C), suggesting that the grana stacks are enriched in PSII. Armond and Arntzen (3) demonstrated that 90% of the PSII reaction centers were localized in the grana stacks with the remainder in the stroma lamellae, which is consistent with our cytochemical data. Previous studies of PSII localizations utilizing ferricyanide as an electron acceptor (12, 17) are unacceptable because ferricyanide is reduced by both PSI and PSII (13). Although Imaizumi and Hiraoka (6) report that a monotetrazole, 2-(2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl)tetrazolium, is reduced at an internal membrane site, these localizations are probably unrelated to electron flow from PSII to PSI because this tetrazolium is not reduced by photosynthetic reactions (9). As in our study, results of Marty (15) indicate that TCNBT reduction occurs on the stroma side of the thylakoid and is concentrated in the partition region of the grana stacks. Vaughn and Duke (20) previously reported that the donor side of PSII, as localized by oxidation of TMBZ, is primarily localized in the grana lamellae.

Immunocytochemistry. The PAP procedure was used in both postembedding and pre-embedding procedures to detect the presence of the P_{700} Chl *a* protein, the reaction center protein of PSI. In the postembedding reaction, all cellular sites are open and exposed for reaction with the antibody whereas in the pre-embedding reaction, the various reagents of the PAP reaction must pass through the cellular membranes in order to react. Positive localizations of the P_{700} Chl *a* protein were obtained using both procedures. Sections exposed to the PAP reagents revealed a strong reaction in both grana and stroma lamellae (Fig. 2A). Controls, in which pre-immune serum was substituted for the P_{700} Chl *a* protein-specific serum, were unstained (Fig. 2B). Strong positive reactions were also noted on isolated thylakoids after incubation in the PAP reagents (Fig. 2C), although no reaction was noted when pre-immune serum was substituted for the P_{700} Chl *a* protein-specific antisera (Fig. 2D). The isolated thylakoids shown in Figure 2C appear to react uniformly and strongly with the antiserum, confirming both the PSI cytochemistry and the postembedding staining with the PAP reagent.

CONCLUSIONS

Our interpretation of the localization of the cytochemical and immunocytochemical reactions are summarized in Figure 3. This model is based upon previous models of the organization of the photosynthetic apparatus (4, 16) and on the localizations obtained here (Figs. 1 and 2) and in a previous study (20). Although it is generally believed that both stroma lamellae and grana lamellae contain PSI (4, 5, 15, 25), some have argued that PSI is confined to the stroma lamellae and the ends of the grana stack (1, 2). Our cytochemical and immunocytochemical data indicate that the P_{700} Chl *a* protein and its associated activity (DAB oxidation) are present in both the stroma and grana lamellae. Although neither the cytochemical or immunocytochemical procedures are strictly quantitative, the presence of PSI activity (Fig.

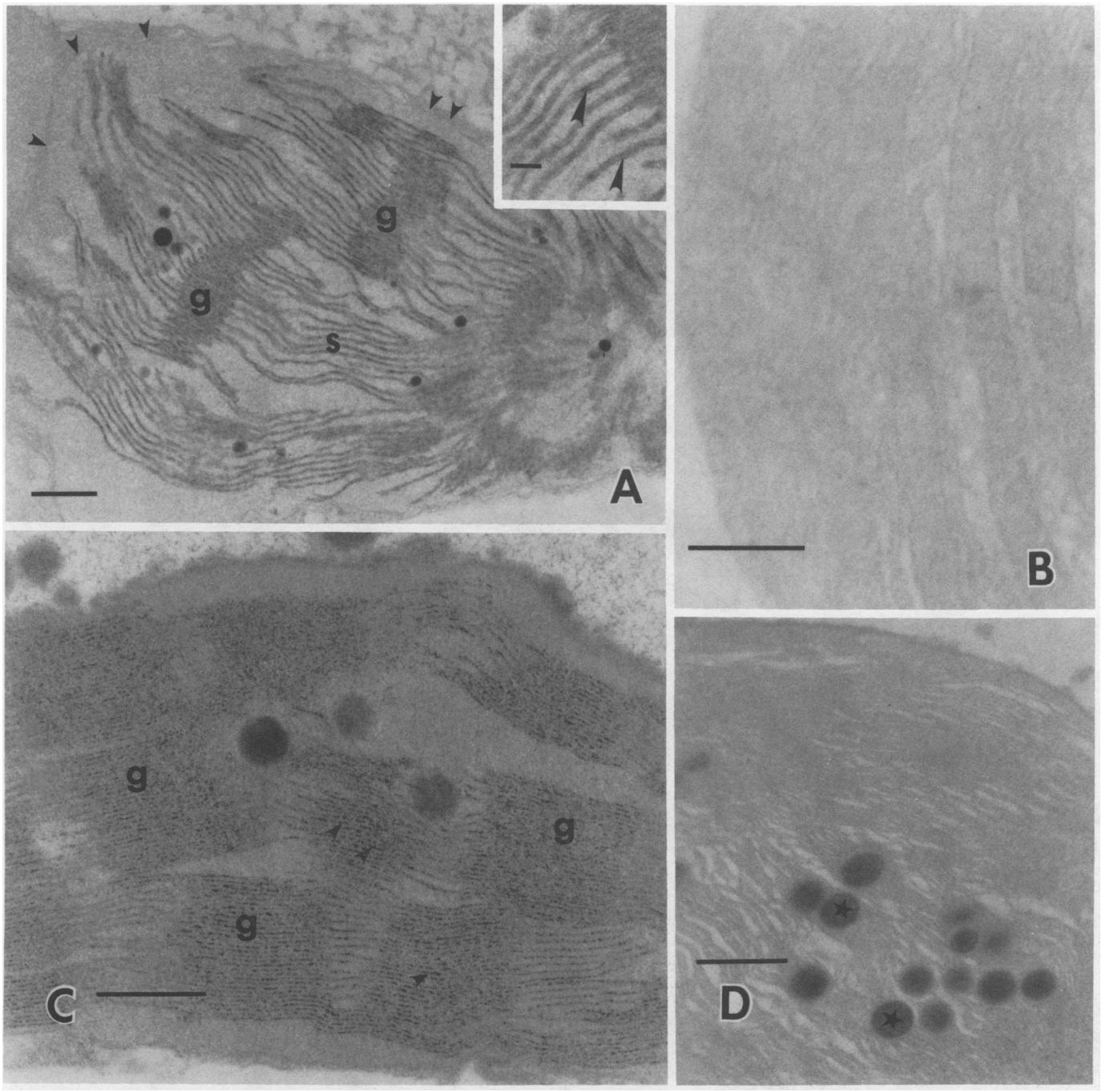

FIG. 1. Cytochemical reactions of barley mesophyll chloroplasts. A, Photooxidation of DAB is observed in both the grana (g) and stroma (s) lamellae. No reaction is noted along the plastid envelope (arrows). At higher magnification (inset), it appears that the DAB products accumulate along the lumen side of the thylakoid (arrows). B, No reaction is noted when tissues are incubated in DAB in the dark. C, TCNBT formazan (arrows) accumulates in the partition region of grana stacks (g) and less densely along the stroma lamellae. D, No TCNBT reduction occurs when 10^{-6} M DCMU is added to the media, indicating the specificity of the reaction for PSII. Plastoglobuli (star) have the same electron density as in C. Bar = 0.5 μm and 0.1 μm in A, inset.

1, A and B) strongly suggests that the P_{700} Chl *a* protein detected (Fig. 2, A and C) is functional in the grana stacks. The presence of PSII in the stroma lamellae (Fig. 1C) and PSI in the grana lamellae (Figs. 1A, 2A, and 2C) may allow for spillover. Several mutants which are completely devoid of stroma lamellae possess high PSI activity (11), which suggests that stroma lamellae are not required for PSI activity as is required in the Anderson model (1, 2). It is demonstrated that most of the TCNBT formazan is deposited in the grana stacks (Fig. 1C), with much less intense depositions on the stroma lamellae. Material prepared for localization of the donor sites of PSII using the TMBZ reagent also indicates a predominant grana localization of PSII activity (20). Thus, if one compares the relative distribution of the two photosystems based upon Chl (1, 2), it is not surprising that one would find relatively less PSI in the grana because of the skewed distribution of PSII.

The model of thylakoid organization of Anderson (1, 2) is based upon the phase separation of appressed from nonappressed membranes. Although we have no reason to doubt the quantitation of P_{700} Chl *a* protein via these procedures, it is possible

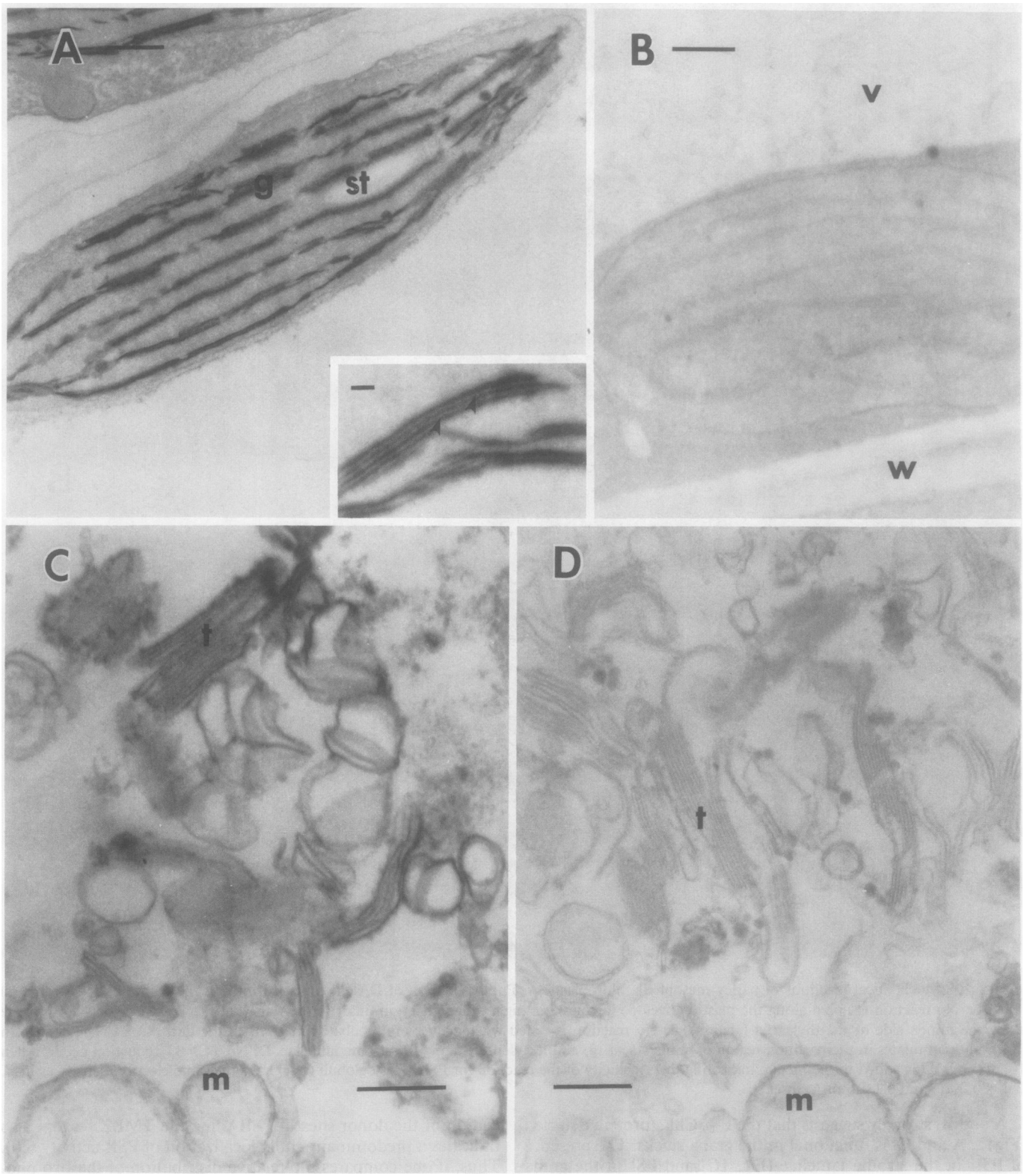

FIG. 2. PAP immunocytochemistry of the P_{700} chlorophyll *a* protein. A, Thylakoids in both grana (g) and stroma lamellae are heavily stained by the PAP reagents. Inset shows higher magnification of individual granum. Partitions (arrows) are not stained. B, Control, substituting pre-immune sera for the P_{700} Chl *a* protein-specific antisera. No reaction is evident on the thylakoids. st = starch; v = vacuole; w = cell wall. C, Isolated thylakoids (t) incubated in the P_{700} Chl *a* protein-specific antisera are intensely stained by the pre-embedding procedure. No reaction is observed in mitochondria (m) that contaminate the thylakoid pellet. D, Control, substituting pre-immune sera for the P_{700} Chl *a* protein-specific antisera. No reaction is evident on the thylakoids (t) or on the mitochondria (m). Bar = 0.5 μm and 0.1 μM in A, inset.

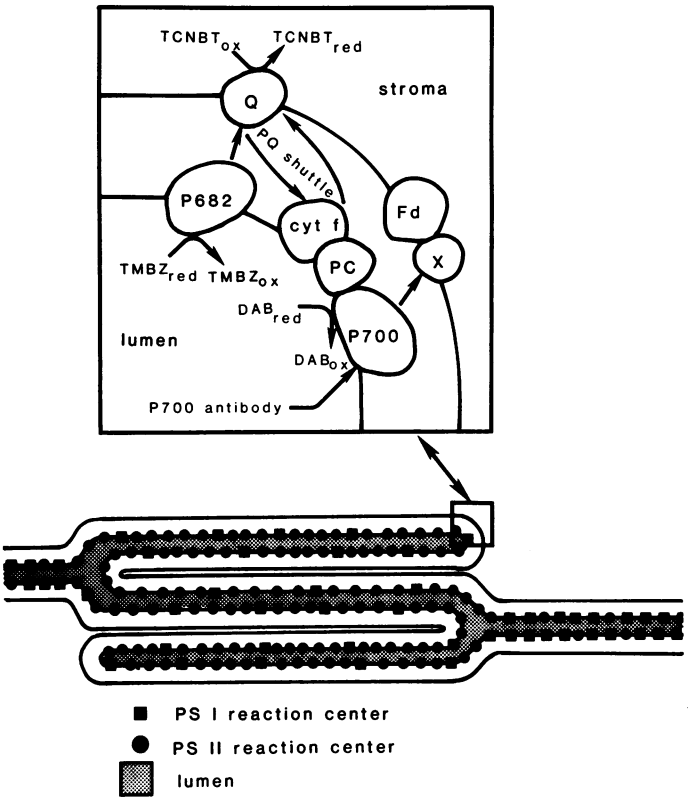

FIG. 3. Diagrammatic interpretation of the spatial organization of PSI and PSII in the thylakoid membranes. P_{682} = reaction center complex of PSII; P_{700} = reaction center complex of PSI; PQ = plastoquinone; PC = plastocyanin; X = ferredoxin-reducing substance; Fd = ferredoxin; Q = primary acceptor of PSII; cyt f = cytochrome *f*. Partial cytochemical reactions are abbreviated as in the text.

that the procedures themselves may not have separated the appressed from the nonappressed lamellae but rather the PSI components from the PSII components. In fact, careful examination of the data obtained from these fractionation studies suggest that at least 20% of the P_{700} Chl *a* protein is found in the appressed lamellae fraction (1, 2). Biological and environmental variations could account for some of the discrepancy between our results and those of Anderson and colleagues (1, 2).

In summary, our cytochemical and immunocytochemical studies establish the presence of the P_{700} Chl *a* protein PSI and activity associated with this photosystem in both grana and stroma lamellae and show that the localization of PSII is preponderantly, but not exclusively, in the grana lamellae. These and other (20) findings, based on partial reactions of the two photosystems and immunocytochemical localizations of the P_{700} Chl *a* protein, have allowed for the construction of a model of the distribution and locations of the reaction centers within the membranes of the photosynthetic apparatus (Fig. 3).

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